Journal of Ethnopharmacology 141 (2012) 1058–1070

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Efficacy of *Daphne oleoides* subsp. *kurdica* used for wound healing: Identification of active compounds through bioassay guided isolation technique

Ipek Süntar[a], Esra Küpeli Akkol[a,*], Hikmet Keles[b], Erdem Yesilada[c], Satyajit D. Sarker[d], Randolph Arroo[e], Turhan Baykal[a]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Etiler 06330, Ankara, Turkey
[b] Department of Pathology, Faculty of Veterinary Medicine, Afyon Kocatepe University, 03200 Afyonkarahisar, Turkey
[c] Department of Pharmacognosy, Faculty of Pharmacy, Yeditepe University, Atasehir 34755, Istanbul, Turkey
[d] Department of Pharmacy, School of Applied Sciences, University of Wolverhampton, MA Building, Wulfurna Street, Wolverhampton WV1 1LY, United Kingdom
[e] Leicester School of Pharmacy, De Montfort University, The Gateway, Leicester LE1 9BH, United Kingdom

ARTICLE INFO

Article history:
Received 31 January 2012
Received in revised form 10 March 2012
Accepted 3 April 2012
Available online 10 April 2012

Keywords:
Anti-inflammatory
Antioxidant
Collagenase
Daphne
Elastase
Excision wounds
Hyaluronidase
Incision wounds
Tensiometer
Thymelaeaceae
Wound healing

ABSTRACT

Ethnopharmacological relevance: In Turkish traditional medicine, the aerial parts of *Daphne oleoides* Schreber subsp. *kurdica* (DOK) have been used to treat malaria, rheumatism and for wound healing.

The aim was to evaluate the ethnopharmacological usage of the plant using *in vivo* and *in vitro* pharmacological experimental models, and to perform bioassay-guided fractionation of the 85% methanolic extract of DOK for the isolation and identification of active wound-healing component(s) and to elucidate possible mechanism of the wound-healing activity.

Materials and methods: In vivo wound-healing activity was evaluated by the linear incision and the circular excision wound models. Anti-inflammatory and antioxidant activities, which are known to support the wound healing process, were also assessed by the Whittle method and the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging assays, respectively. The total phenolic content of the extract and subextracts was estimated to establish any correlation between the phenolic content and the antioxidant activity. The methanolic extract of DOK was subjected to various chromatographic separation techniques leading to the isolation and identification of the active component(s). Furthermore, *in vitro* hyaluronidase, collagenase and elastase enzymes inhibitory activity assays were conducted on the active components to explore the activity pathways of the remedy.

Results: After confirmation of the wound-healing activity, the methanolic extract was subjected to successive solvent partitioning using solvents of increasing polarity creating five subextracts. Each subextract was tested on the same biological activity model and the ethyl acetate (EtOAc) subextract had the highest activity. The EtOAc subextract was subjected to further chromatographic separation for the isolation of components **1**, **2** and **3**. The structures of these compounds were elucidated as daphnetin (**1**), demethyldaphnoretin 7-*O*-glucoside (**2**) and luteolin-7-*O*-glucoside (**3**). Further *in vivo* testing revealed that luteolin-7-*O*-glucoside was responsible for the wound-healing activity of the aerial parts. It was also found to exert significant anti-inflammatory, antioxidant, anti-hyaluronidase and anti-collagenase activities.

Conclusion: The present study explored the wound-healing potential of *Daphne oleoides* subsp. *kurdica*. Through bioassay-guided fractionation and isolation techniques, luteolin-7-*O*-glucoside was determined as the main active component of the aerial parts. This compound exerts its activity through inhibition of hyaluronidase and collagenase enzymes activity as well as interfering with the inflammatory stage.

1. Introduction

The *Daphne* L. species (Thymelaeaceae) are evergreen shrubs native to Asia, Europe, and North Africa. Among the 70 species distributed worldwide seven species grow in Turkey, namely, *Daphne glomerata*, *Daphne gnidioides*, *Daphne mezereum*, *Daphne mucronata*, *Daphne oleoides*, *Daphne pontica* and *Daphne sericea* (Tan, 1982). There are two additional subspecies of *Daphne oleoides*, subsp. *oleoides* and subsp. *kurdica*.

Various parts of *Daphne* species have been used in traditional medicines worldwide to treat rheumatic pain or gastric pain, as a purgative and an abortificient (Ronlan and Wickberg, 1970). The

* Corresponding author. Tel.: +90 312 2023185; fax: +90 312 2235018.
E-mail address: esrak@gazi.edu.tr (E. Küpeli Akkol).

0378-8741/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jep.2012.04.001

barks and fruits of *Daphne mezereum* have been used as a remedy for the treatment of ulcers, rheumatism and some skin diseases in traditional medicines (Grieve and Leyel, 1967). In Turkish folk medicine, the powdered root of *Daphne oleoides* mixed with sugar has been used against malaria. An ointment prepared from the aerial parts with butter has been used topically for the treatment of rheumatic pain and for wound healing (Fujita et al., 1995; Yesilada et al., 1995). The stem bark of *Daphne pontica* has been used against diarrhea (Yesilada et al., 1999), while *Daphne mezereum* was prescribed as a purgative (Baytop, 1999). The poultice prepared by cooking the leaves and the aerial parts of *Daphne gnidioides* with *Origanum onites* has been prescribed against stomachache.

Phytochemical studies have revealed that *Daphne* species contain a wide range of phytochemicals including flavonoids, coumarins, lignans, sesquiterpenes, diterpenes, triterpenes and steroids (Murray et al., 1982; Baba et al., 1986; Ulubelen et al., 1986; Kreher et al., 1990; Niwa et al., 1991; Ullah et al., 1998; Taninaka et al., 1999; Ullah, 1999; Okunishi et al., 2002). Various biological activities were also reported for *Daphne* species including analgesic, anti-inflammatory, antioxidant, anticancerogenic, antimicrobial, anti-ulcerogenic, abortive, hypocholesterolemic and hemostatic (Murakami et al., 1992; Cottiglia et al., 2001; Hong et al., 2002; Deiana et al., 2003; Kupeli et al., 2007; Zhang et al., 2007; Zheng et al., 2007; Lee et al., 2009).

The present research aimed to evaluate the efficiency of *Daphne oleoides* subsp. *kurdica* aerial parts as a wound-healing remedy in Turkish folk medicine. After demonstration of *in vivo* wound-healing activity, the 85% methanolic extract will be subjected to successive *in vivo* bioassay-guided fractionation for the isolation of active component. Afterwards *in vivo* and *in vitro* techniques will also be applied to study the possible activity mechanism.

2. Materials and methods

2.1. Plant material

The aerial parts of *Daphne oleoides* subsp. *kurdica* were collected from Bolkar Mountains, Meydan Plateau, Adana-Turkey in July, 2009.

The voucher specimen of the plant was authenticated by Prof. Dr. Hayri Duman from Gazi University, Department of Biology, Faculty of Science and Art, Ankara) and specimen of the plant (GUE 2975) was deposited in the Herbarium of Faculty of Pharmacy, Gazi University, Ankara, Turkey. The aerial parts were shade dried and ground using a blender.

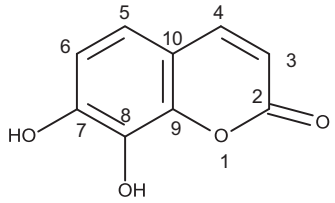

Fig. 1. Chemical structure of the compound **1** (daphnetin).

2.2. Extraction, fractionation and isolation procedures for the bioassays

Dried and powdered aerial parts (1000 g) of *Daphne oleoides* subsp. *kurdica* were extracted with 85% MeOH and evaporated to dryness to give "DOK-MeOH" (yield: 20.6%). The residual dried extract was then dissolved in 400 mL of MeOH/H_2O (9:1) extracted with *n*-hexane (20 × 500 mL). Combined *n*-hexane subextracts were evaporated under reduced pressure to give "DOK-*n*-hexane" (yield: 5.3%). Methanol was removed from the remaining extract and diluted with distilled H_2O to 400 mL and successively extracted with dichloromethane (20 × 500 mL) and EtOAc (20 × 500 mL). Each solvent extract was evaporated to dryness under reduced pressure to give "DOK-CH_2Cl_2" (yield: 7.2%) and "DOK-EtOAc" (yield: 8.1%), respectively. The remaining aqueous extract was further extracted with *n*-butanol saturated with water (20 × 500 mL) and evaporated to dryness at 40 °C under reduced pressure to yield "DOK-*n*-BuOH" (yield: 27.3%). The final aqueous phase was also evaporated to dryness "DOK-R-H_2O" (yield: 15.2%).

2.2.1. Fractionation of DOK-EtOAc extract

Following bioassay-guided procedures DOK-EtOAc, the active subextract, was further fractionated by chromatographic techniques. DOK-EtOAc (2 g) was subjected to chromatographic separation on Silica gel column using $CHCl_3$ and $CHCl_3$:MeOH (95:5) and $CHCl_3$:MeOH (90:10) as eluents, and fractions of 10 mL were collected. The collected fractions were combined based on their TLC patterns as follows: Fr. (1–8) (169.7 mg), Fr. (9–15) (122.3 mg), Fr. (16–21) (153.5 mg) and Fr. (22–39) (1034.5 mg).

2.2.2. Chromatographic separation and isolation of the active constituents

Fraction (19–27) was further subjected to High Pressure Liquid Chromatography (HPLC, Dionex Ultimate 3000 HPLC, PN: 5722.0025 used with Phenomenex column, Luna 5μ C_{18}, 150 × 10 mm) to yield compounds **1** (24.5 mg), **2** (12.5 mg) and **3** (119.8 mg) (Figs. 1–3). The chromatographic separation of the fraction was performed using a gradient elution program. Mobile

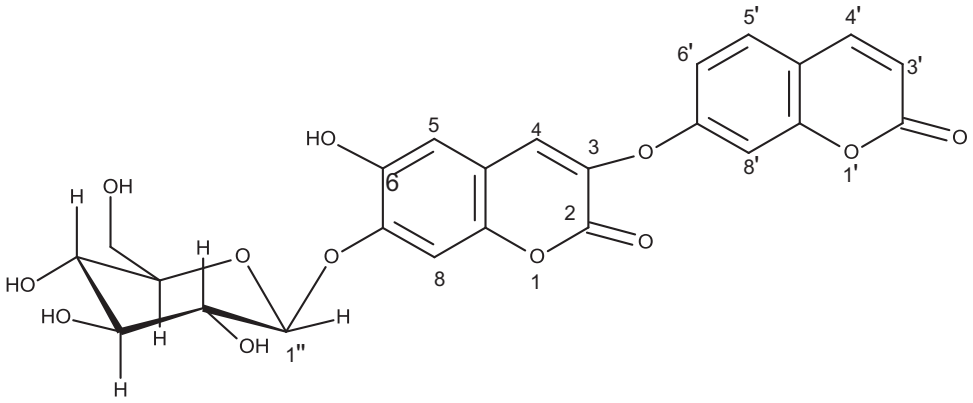

Fig. 2. Chemical structure of the compound **2** (demethyldaphnoretin-7-*O*-glucoside).

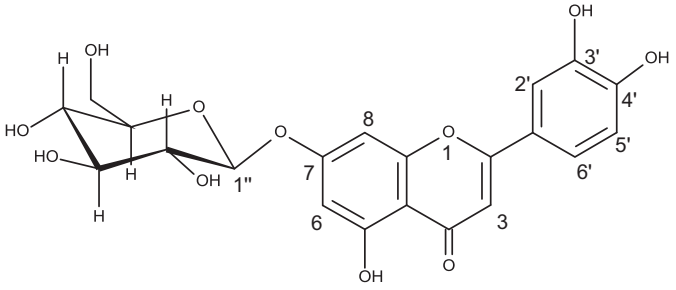

Fig. 3. Chemical structure of the compound **3** (luteolin-7-*O*-glucoside).

phase, solvent A: HPLC grade water (H_2O); solvent B: (MeOH); Mode: gradient, increasing the organic phase (MeOH) from 30 to 100% over 30 min, flow rate: 2 mL/min; injected volume: 200 μL. Detection wavelength of 220 nm on Channel A, 254 nm on Channel B, 280 nm on Channel C and 331 nm on Channel D. After the end of each run, a delay time of 10 min then modifying B to 30% and a second delay for 15 min before the next injection was necessary in order to equilibrate the column.

2.2.3. Structure elucidation of the compounds

Nuclear magnetic resonance (^{1}H and ^{13}C NMR) and mass spectral (MS) techniques were used for the structure elucidation of the compounds. NMR spectra were recorded on a Bruker spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) instrument, and using MeOD as solvent. FT-MS analyses were performed using a Finnigan spectrometer. The isolates were identified as daphnetin (**1**), demethyldaphnoretin-7-*O*-glucoside (**2**) and luteolin-7-*O*-glucoside (**3**) by comparison of their spectroscopic data with those of published in related references (Riaz et al., 2002; Ma et al., 2007; Gohari et al., 2011).

Daphnetin: FABMS (+ve ion mode): *m*/*z* 379 $[2M+Na]^+$, 201 $[M+Na]^+$. ^{1}H NMR: δ 7.78 (1H, d, J = 9.6 Hz, H-4), 7.13 (1H, d, J = 8.8 Hz, H-5), 6.78 (1H, d, J = 8.8 Hz, H-6), 6.12 (1H, d, J = 9.6 Hz, H-3). ^{13}C NMR: δ 163.5 (C-2), 151.1 (C-7), 146.7 (C-8a), 144.9 (C-4), 133.5 (C-8), 120.2 (C-5), 113.9 (C-6), 113.8 (C-4a), 112.2 (C-3).

Demethyldaphnoretin-7-O-glucoside: FABMS (−ve ion mode): *m*/*z* 999 $[2M-H]^-$, 499 $[M-H]^-$. ^{1}H NMR: δ 7.95 (1H, d, J = 9.6 Hz, H-4′), 7.70 (1H, s, H-4), 7.65 (1H, d, J = 8.8 Hz, H-5′), 7.45 (1H, s, H-5), 7.15 (1H, dd, J = 8.8 Hz; 2.5 Hz, H-6′), 7.05 (1H, d, J = 2.5 Hz, H-8′), 6.90 (1H, s, H-8), 6.36 (1H, d, J = 9.6 Hz, H-3′), 5.10 (1H, d, J = 7.7 Hz, H-1 of glucose), 3.30–3.80 (6H, m, glucose protons). ^{13}C NMR: δ 160.4 (C-2′), 160.2 (C-2), 158.7 (C-7′), 155.8 (C-8a′), 151.3 (C-7), 149.7 (C-8a), 145.4 (C-6), 144.7 (C-4′), 136.1 (C-3), 130.9 (C-4), 130.5 (C-5′), 116.7 (C-4a′), 114.9 (C-3′), 112.6 (C-6′), 112.5 (C-4a), 111.6 (C-5), 104.8 (C-8′), 104.6 (C-8), 103.2 (C-1 of glucose), 78.5 (C-5 of glucose), 77.6 (C-3 of glucose), 74.8 (C-2 of glucose), 71.1 (C-4 of glucose), 61.1 (C-6 of glucose).

Luteolin-7-O-glucoside: FABMS (+ve ion mode): *m*/*z* 449 $[M+H]^+$. ^{1}H NMR: δ 7.41 (1H, dd, J = 8.0 Hz; 1.8 Hz, H-6′), 7.40 (1H, d, J = 1.8 Hz, H-2′), 6.60 (1H, s, H-3), 6.90 (1H, d, J = 8 Hz, H-5′), 6.80 (1H, d, J = 1.8 Hz, H-8), 6.46 (1H, d, J = 1.8 Hz, H-6), 5.12 (1H, d, J = 7.8 Hz, H-1 of glucose), 3.20–3.90 (6H, m, glucose protons). ^{13}C NMR: δ 181.5 (C-4), 164.4 (C-2), 162.3 (C-7), 161.0 (C-5), 156.3 (C-9), 149.5 (C-4′), 145.4 (C-3′), 121.0 (C-1′), 118.9 (C-6′), 115.5 (C-5′), 113.3 (C-2′), 105.0 (C-10), 102.8 (C-3), 99.5 (C-6), 99.1 (C-1 of glucose), 94.5 (C-8), 77.1 (C-5 of glucose), 76.2 (C-3 of glucose), 72.9 (C-2 of glucose), 69.2 (C-4 of glucose), 60.3 (C-6 of glucose).

2.3. Determination of total phenolic content of the extract and subextracts

Total phenolic contents of the methanolic extract and subextracts were quantified by using the reference methods involving the Folin–Ciocalteu reagent and gallic acid as standard compounds (Spanos and Wrolstad, 1990). An aliquot of extract solution (100 μL) containing 1 mg extract was taken into a volumetric flask, distilled water and the Folin–Ciocalteu reagent were added and flask was shaken thoroughly. Na_2CO_3 (4 mL) was added and the mixture was allowed to stand for 2 h with intermittent shaking at room temperature. Then absorbance was measured at 765 nm. The same procedure was applied to standard gallic acid solutions prepared in different concentrations (0.05 mg/mL; 0.1 mg/mL; 0.15 mg/mL; 0.25 mg/mL and 0.5 mg/mL) to prepare the standard curve.

2.4. Pharmacological experiments

2.4.1. In vivo biological activity tests

2.4.1.1. Animals. Male Sprague-Dawley rats (160–180 g) and Swiss albino mice (20–25 g) purchased from the animal breeding laboratory of Saki Yenilli (Ankara, Turkey) were used in the experiments.

The animals were left for 3 days for acclimatization into animal room conditions and were maintained on standard pellet diet and water *ad libitum*. A minimum of six rats were used in each group for wound healing experiments, while 10 mice were used in anti-inflammatory studies. The present study was performed according to the international rules considering the animal experiments and biodiversity rights (Gazi University Ethical Council Project Number: G.U.ET-10.027).

2.4.1.2. Preparation of test samples for bioassay. For the anti-inflammatory test model, samples were given orally to test animals after suspending in a mixture of distilled water and 0.5% sodium carboxymethyl cellulose (CMC). The control group of animals received the same experimental handling as those of the test groups except that the drug treatment was replaced with appropriate volumes of the dosing vehicle. Indomethacin (10 mg/kg) in 0.5% CMC was used as a reference drug.

For the assessment of wound-healing activity by using the incision and the excision wound models, an ointment prepared with the test materials was topically applied onto the wounded area on the dorsal part of test animals. The test ointments were prepared by mixing either extracts/subextract/fractions or compounds with a mixture of ointment base consisting of glycol stearate:propylene glycol and liquid paraffin (3:6:1) in a mortar thoroughly. Treatments were started immediately after the production of wound by daily application of the test ointments on the wounded area. The control group animals were topically treated with blank vehicle base consisting of glycol stearate:propylene glycol:liquid paraffin (3:6:1) mixture, while the animals in negative control group were not treated with any product. Madecassol® (Bayer, 00001199) (0.5 g) was used topically as the reference drug.

2.4.1.3. Wound-healing activity.

2.4.1.3.1. Linear incision wound model. Animals, six rats in each group, were anesthetized with 0.15 cc Ketasol® (Richterpharma). The hairs on the dorsal part of the rats were shaved and cleaned with 70% alcohol. Two 5 cm-length linear-paravertebral incisions were made with a sterile blade through the shaved skin at the distance of 1.5 cm from the dorsal midline on each side. Three surgical sutures were placed each 1 cm apart.

The ointments prepared with test samples, the reference drug (Madecassol®) or ointment base [glycol stearate:propylene glycol:liquid paraffin (3:6:1)] were topically applied on the dorsal wounds in each group of animals once daily throughout 9 days. All the sutures were removed on the last day and tensile strength of previously wounded and treated skin was measured by using a

tensiometer (Zwick/Roell Z0.5, Germany) (Suguna et al., 2002; Lodhi et al., 2006).

2.4.1.3.2. Circular excision wound model. This model was used to monitor wound contraction and wound closure time. Each group of animals (seven animals in each) was anesthetized by 0.01 cc Ketasol® (Richterpharma). The back hairs of the mice were depilated by shaving. The circular wound was created on the dorsal interscapular region of each animal by excising the skin with a 5 mm biopsy punch (Nopa instruments, Germany); wounds were left open (Tramontina et al., 2002). Test samples, the reference drug (Madecassol®, Bayer) and the vehicle ointments were applied topically once a day till the wound was completely healed. The progressive changes in wound area were monitored by a camera (Fuji, S20 Pro, Japan) every other day. Later on, wound area was evaluated by using AutoCAD program. Wound contraction was calculated as percentage of the reduction in wounded area. A specimen sample of tissue was isolated from the healed skin of each group of mice for the histopathological examination (Süntar et al., 2011).

2.4.1.3.3. Histopathology. The skin specimens from each group were collected at the end of the experiment (on day 12). Samples were fixed in 10% buffered formalin, processed and blocked with paraffin and then sectioned into 5-μm sections and stained with hematoxylin & eosin (HE) and Van Gieson (VG) stains. The tissues were examined by light microscope (Olympus CX41 attached Kameram® Digital Image Analyze System) and graded as mild (+), moderate (++) and severe (+++) for epidermal or dermal re-modeling. Re-epithelialization or ulcus in epidermis; fibroblast proliferation, mononuclear and/or polymorphonuclear cells, neo-vascularization and collagen depositions in dermis were analyzed to score the epidermal or dermal re-modeling. Van Gieson stained sections were analyzed for collagen deposition. At the end of the examination, all the wound healing processes were combined and staged for wound healing phases as inflammation, proliferation, and re-modeling in all groups.

2.4.1.3.4. Hydroxyproline estimation. Tissues were dried in hot air oven at 60–70 °C until consistent weight was achieved. Afterwards, samples were hydrolyzed with 6 N HCl for 3 h at 130 °C. The hydrolyzed samples were adjusted to pH 7 and subjected to chloramin T oxidation. The colored adduct formed with Ehrlich reagent at 60 °C was read at 557 nm. Standard hydroxyproline was also run and values reported as μg/mg dry weight of tissue (Degim et al., 2002).

2.4.1.4. Anti-inflammatory activity.

2.4.1.4.1. Acetic acid-induced increase in capillary permeability. Effect of the test samples on the increased vascular permeability induced by acetic acid in mice was determined according to Whittle method (Whittle, 1964) with some modifications (Yesilada and Küpeli, 2007). Each test sample was administered orally to a group of 10 mice in 0.2 mL/20 g body weight. Thirty minutes after the administration, tail of each mice was injected with 0.1 mL of 4% Evans blue in saline solution (i.v.) and waited for 10 min. Then, 0.4 mL of 0.5% (v/v) AcOH was injected i.p. After 20 min incubation, the mice were killed by dislocation of the neck, and the viscera were exposed and irrigated with distilled water, which was then poured into 10 mL volumetric flasks through glass wool. Each flask was made up to 10 mL with distilled water, 0.1 mL of 0.1 N NaOH solution was added to the flask, and the absorption of the final solution was measured at 590 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA). A mixture of distilled water and 0.5% CMC was given orally to control animals, and they were treated in the same manner as described above.

2.4.2. In vitro biological activity tests

2.4.2.1. Antioxidant activity.

2.4.2.1.1. The DPPH radical scavenging assay. The hydrogen atom or electron donation capacity of the corresponding extracts was computed from the bleaching property of the purple-colored MeOH solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The samples and reference dissolved in MeOH were mixed with DPPH solution (80 μg/mL). Remaining DPPH amount was measured at 517 nm using spectrophotometer. The results were compared to that of quercetin employed as the reference.

DPPH inhibition activities were calculated according to the following formula:

$$\text{Inhibition}\ (\%) = \frac{(A_{\text{control}} - A_{\text{sample}}) \times 100}{A_{\text{control}}}$$

where A_{control} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts/reference. Experiments were run in duplicate and the results were conveyed as inhibition values (Kumarasamy et al., 2003).

2.4.2.2. Determination of hyaluronidase inhibitory activity. The inhibition of hyaluronidase was assessed by the measurement of the amount of *N*-acetylglucosamine released from sodium hyaluronate (Lee and Choi, 1999; Sahasrabudhe and Deodhar, 2010). 50 μL of bovine hyaluronidase (7900 units/mL) was dissolved in 0.1 M acetate buffer (pH 3.6). Then this solution was mixed with 50 μL of different concentrations of the oils dissolved in 5% DMSO. For the control group 50 μL of 5% DMSO was added instead of the oils. After 20 min incubation at 37 °C, 50 μL of calcium chloride (12.5 mM) was added to the mixture and again incubated for 20 min at 37 °C. 250 μL sodium hyaluronate (1.2 mg/mL) was added and incubated for 40 min at 37 °C. After incubation the mixture was treated with 50 μL of 0.4 M NaOH and 100 μL of 0.2 M sodium borate and then incubated for 3 min in boiling water bath. 1.5 mL of *p*-dimethylaminobenzaldehyde solution was added to the reaction mixture after cooling to room temperature and was incubated at 37 °C for 20 min to develop a color. The absorbance was measured at 585 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA).

2.4.2.3. Determination of collagenase inhibitory activity. The samples were dissolved in DMSO. The sample solution and *Clostridium histolyticum* (ChC) were dissolved in 50 mM Tricine buffer (with 0.4 M NaCl and 0.01 M $CaCl_2$, pH 7.5) and preincubated at 25 °C for 5 min. Then, 2 mM *N*-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) was prepared in the same buffer. 25 μL buffer, 25 μL test sample and 25 μL enzyme were added to each well and incubated for 15 min. 50 μL substrate was added to the mixture to immediately measure the decrease of the optical density (OD) at 340 nm using spectrometer.

The ChC inhibition activities were calculated according to the following formula:

$$\text{ChC inhibition activity}\ (\%) = \frac{(\text{OD}_{\text{Control}} - \text{OD}_{\text{Sample}}) \times 100}{\text{OD}_{\text{Control}}}$$

where $\text{OD}_{\text{control}}$ and $\text{OD}_{\text{sample}}$ represent the optical densities in the absence and presence of sample, respectively (Barrantes and Guinea, 2003).

2.4.2.4. Determination of elastase inhibitory activity. The sample solution and human neutrophil elastase enzyme (HNE) (17 mU/mL) were mixed in 0.1 M Tris–HCl buffer (pH 7.5), then incubated at 25 °C for 5 min. *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (MAAPVN) was added to the mixture and incubated at 37 °C for 1 h.

Table 1
Effects of the extract and sub-extracts from *Daphne oleoides* subsp. *kurdica* on linear incision wound model.

Material	Dose (%)	Tensile strength ± S.E.M.	(%Tensile strength)
Vehicle		14.26 ± 2.01	7.87
Negative control		13.22 ± 2.47	–
DOK-MeOH	1	19.90 ± 2.14	39.55**
DOK-*n*-hexane	1	15.63 ± 2.20	9.61
DOK-CH_2Cl_2	1	15.80 ± 1.49	10.79
	1	19.44 ± 1.63	36.33**
DOK-EtOAc	3	19.69 ± 1.18	38.08**
	5	19.31 ± 1.26	35.41**
DOK-*n*-BuOH	1	18.78 ± 1.38	31.69*
DOK-R-H_2O	1	16.04 ± 1.89	12.48
Madecassol®	1	21.40 ± 1.15	50.07***

Percentage of the tensile strength values: the vehicle group was compared to the negative control group. The extracts and the reference material were compared to vehicle group; S.E.M., standard error of the mean.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Afterwards, the reaction was stopped by the addition of soybean trypsin inhibitor (1 mg/mL) and the optical density due to the formation of *p*-nitroaniline was immediately measured at 405 nm. The HNE inhibition activities were calculated as in the ChC inhibition activity (Melzig et al., 2001).

2.4.3. Statistical analysis of the data

Data obtained from animal experiments were expressed as the mean standard error (±S.E.M.). Statistical differences between the treated and the control groups were evaluated by ANOVA and Students–Newman–Keuls *post hoc* tests. The values of $p \leq 0.05$ were considered statistically significant.

Histopathologic data were considered to be nonparametric; therefore, no statistical tests were performed.

3. Results and discussion

Wounds are physical injuries that result in a break in the epithelial integrity of the skin and usually caused by disruption of the structure and function of the normal tissue. Proper wound-healing is essential for the restoration of the anatomical continuity and functional status of the skin (Kumar et al., 2007). On the other hand, particularly metabolic diseases may disrupt the regenerative process causing delayed healing. This brings out a financial burden in the developed and developing countries. Therefore, the possibility of discovering the effective therapies from traditional medicines has been explored (Krishnan, 2006). A large number of plants and plant extracts have been used in traditional medicines worldwide for the treatment of cuts, wounds, and burns. Previously reported ethnobotanical field surveys have reported that *Daphne oleoides* subsp. *kurdica* has been frequently used for wound-healing by the people in the rural areas (Fujita et al., 1995; Yesilada et al., 1995).

The *in vivo* wound-healing potential of the 85% methanolic extract of the aerial part of DOK was investigated in the present study. One percent ointment formulation prepared from the extract exerted a significant wound healing activity on the linear incision and the circular excision wound models. MeOH extract was submitted to successive solvent extractions with water immiscible organic solvents of increasing polarity. Wound-healing activity of each solvent extract, *i.e.*, *n*-hexane, methylene chloride, EtOAc, *n*-butanol subextracts and the residual aqueous subextract were then investigated using the same wound models. As shown in Tables 1 and 2, EtOAc and *n*-BuOH subextracts

Table 2
Effects of the extract and sub-extracts from *Daphne oleoides* subsp. *kurdica* on circular excision wound model.

Material	Dose (%)	Wound area (mm^2) ± S.E.M. (contraction%)						
		Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Vehicle		19.19 ± 2.01	17.03 ± 2.16 (5.18)	14.94 ± 2.27 (7.15)	13.05 ± 1.86 (2.90)	6.37 ± 1.20 (11.40)	4.59 ± 1.67	2.66 ± 0.47 (10.74)
Negative control		19.50 ± 2.25	17.96 ± 2.03	16.09 ± 1.96	13.44 ± 1.51	7.19 ± 1.09	4.36 ± 1.73	2.98 ± 0.62
DOK-MeOH	1	20.26 ± 1.78	15.69 ± 3.37 (7.87)	13.64 ± 1.19 (8.70)	10.68 ± 1.67 (18.16)	4.50 ± 1.04 (29.36)	2.25 ± 0.19 (50.98)**	0.48 ± 0.24 (81.95)***
DOK-*n*-hexane	1	20.14 ± 1.89	15.72 ± 1.53 (7.69)	15.03 ± 2.33	12.29 ± 1.68 (5.82)	6.15 ± 1.59 (3.45)	4.11 ± 0.99 (10.46)	2.17 ± 0.88 (18.42)
DOK-CH_2Cl_2	1	20.01 ± 1.57	15.30 ± 1.19 (10.16)	13.51 ± 1.59 (9.57)	13.47 ± 1.31	5.86 ± 1.15 (8.01)	3.76 ± 0.36 (18.08)	2.19 ± 0.32 (17.67)
	1	19.49 ± 1.88	14.67 ± 1.72 (13.86)	12.37 ± 1.11 (17.20)	9.97 ± 1.10 (23.60)	5.04 ± 0.47 (20.88)	2.97 ± 0.60 (35.29)**	1.07 ± 0.09 (59.77)**
DOK-EtOAc	3	19.56 ± 1.29	14.38 ± 1.94 (15.56)	12.98 ± 1.13 (13.12)	10.22 ± 1.66 (21.69)	5.37 ± 1.10 (15.69)	3.19 ± 0.88 (30.50)*	1.18 ± 0.19 (55.64)**
	5	20.03 ± 1.46	15.57 ± 1.68 (8.57)	13.59 ± 1.57 (9.04)	11.59 ± 1.29 (11.19)	4.79 ± 1.15 (24.80)	3.42 ± 1.53 (25.49)	1.34 ± 0.11 (49.62)**
DOK-*n*-BuOH	1	19.22 ± 1.16	13.96 ± 1.14 (18.03)	13.08 ± 1.54 (12.45)	10.28 ± 1.44 (21.23)	5.13 ± 1.04 (19.47)	3.17 ± 0.23 (30.94)*	1.27 ± 0.14 (52.26)**
DOK-R-H_2O	1	19.25 ± 1.08	15.20 ± 1.97 (10.75)	14.31 ± 1.16 (4.22)	12.07 ± 1.24 (7.51)	5.64 ± 1.27 (11.46)	4.16 ± 0.78 (9.36)	2.14 ± 0.47 (19.55)
Madecassol®	1	19.40 ± 1.19	14.57 ± 1.05 (14.45)	11.09 ± 1.67 (25.77)	5.50 ± 1.03 (57.85)**	1.30 ± 0.35 (79.59)**	0.65 ± 0.08 (85.84)***	0.00 ± 0.00 (100.00)***

Percentage of the contraction values: the vehicle group was compared to the negative control group. The fractions and the reference material were compared to vehicle group; S.E.M., standard of the mean.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 3
Effects of the fractions from DOK-EtOAc on linear incision wound model.

Material	Tensile strength ± S.E.M.	(%Tensile strength)
Vehicle	10.21 ± 1.86	3.76
Negative control	9.84 ± 1.72	–
DOK-Fr. (1–8)	10.94 ± 1.65	7.15
DOK-Fr. (9–15)	10.07 ± 1.92	–
DOK-Fr. (16–21)	11.19 ± 2.19	9.59
DOK-Fr. (22–39)	13.81 ± 1.50	35.26**
Madecassol®	15.74 ± 1.21	54.16***

Percentage of the tensile strength values: the vehicle group was compared to the negative control group; the extracts and the reference material were compared to vehicle group; S.E.M., standard error of the mean.
** $p < 0.01$.
*** $p < 0.001$.

displayed statistically significant wound-healing activity. Due to the higher efficiency of the former following investigations were carried out on the EtOAc subextract. Interestingly, the effect of this extract was not dose-dependent, in fact decreased in higher concentrations.

In order to identify the components in the EtOAc subextract responsible from the wound healing activity, it was submitted to silica gel column chromatography and eluted fractions were grouped into four based on their chemical fingerprinting on TLC examination. DOK-Fr. (22–39) showed the best wound-healing effect with 35.3% increase in tensile strength in the linear incision (Table 3), and 47.9% contraction in the circular excision wound models (Table 4). DOK-Fr. (22–39) was further fractionated by reversed phase-HPLC and two coumarin derivatives and one flavonoid were isolated. The isolated compounds were identified as daphnetin (**1**), demethyldaphnoretin 7-*O*-glucoside (**2**) and luteolin-7-*O*-glucoside (**3**) by spectral analysis. Due to the higher yields of daphnetin and luteolin-7-*O*-glucoside in the active fraction, further biological activity studies were conducted on these compounds. The results demonstrated that luteolin-3-*O*-glucoside possessed significant wound-healing effect with the values of 39.9% in the linear incision (Table 5), and 61.2% in the circular excision wound models (Table 6).

At the end of the experiments, the wounded areas were removed from each animal and tissue sections were evaluated histopathologically. Phases in the healing processes (inflammation, proliferation, and remodeling) were observed and recorded within the experimental groups with different degrees. The histopathological data supported the experimental results of both the linear incision and the circular excision wound models. All the tissues treated with DOK-MeOH, EtOAc subextract, DOK-Fr. (22–39) and luteolin-7-*O*-glucoside ointments demonstrated good healing processes with the capacity of faster re-epithelialization and high collagen concentration compared to the other groups tested. Delayed wound healing processes were observed especially in the vehicle and negative control groups compared to the other groups (Figs. 4–6).

In order to reveal the mechanism of wound-healing activity, several other experiments were also carried out on the extract, subextracts, fractions and active constituent.

Similar activity results were obtained in the hydroxyproline analysis. Tissues treated with DOK-MeOH, EtOAc subextract, DOK-Fr. (22–39) and luteolin-7-*O*-glucoside ointments were found to possess high hydroxyproline content (Tables 7–9).

Due to close relationship between wound-healing process and inflammation, anti-inflammatory activity of the extract, subextracts, fractions and isolates were also investigated by the Whittle method. This model of inflammation

Table 4
Effects of the fractions from DOK-EtOAc on circular excision wound model.

Material	Wound area (mm^2) ± S.E.M. (Contraction%)						
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Vehicle	19.25 ± 2.04	17.23 ± 1.85 (4.49)	16.04 ± 1.31 (1.29)	13.48 ± 1.23 (7.32)	7.54 ± 1.04 (8.94)	4.31 ± 0.49 (10.02)	3.28 ± 0.56 (6.02)
Negative control	19.62 ± 2.27	18.04 ± 1.44	16.25 ± 1.37	13.67 ± 1.13	8.28 ± 1.15	4.79 ± 1.10	3.49 ± 0.56
DOK-Fr. (1–8)	19.43 ± 2.43	17.12 ± 1.35 (0.64)	15.11 ± 1.26 (6.23)	13.15 ± 1.04 (2.45)	7.24 ± 1.18 (3.98)	3.82 ± 0.54 (11.37)	2.99 ± 0.28 (8.84)
DOK-Fr. (9–15)	19.61 ± 2.07	15.85 ± 1.32 (8.01)	13.64 ± 1.21 (14.96)	11.19 ± 1.34 (16.99)	6.07 ± 0.85 (19.49)	3.58 ± 0.19 (16.94)	2.85 ± 0.12 (13.11)
DOK-Fr. (16–21)	19.57 ± 2.19	16.36 ± 1.78 (5.05)	14.28 ± 1.16 (10.97)	12.98 ± 1.18 (7.42)	6.89 ± 1.60 (13.26)	3.50 ± 0.81 (18.79)	2.43 ± 0.56 (25.91)
DOK-Fr. (22–39)	19.89 ± 2.16	16.88 ± 1.73 (2.03)	13.87 ± 1.45 (13.53)	10.54 ± 1.40 (21.81)	6.48 ± 1.17 (14.06)	2.62 ± 0.23 (39.21)*	1.71 ± 0.24 (47.87)**
Madecassol®	19.39 ± 2.11	15.28 ± 1.51 (11.32)	12.16 ± 1.08 (24.19)	8.24 ± 0.78 (38.87)*	3.18 ± 0.14 (57.82)**	1.22 ± 0.11 (71.69)**	0.00 ± 0.00 (100.00)***

Percentage of the contraction values: the vehicle group was compared to the negative control group; the fractions and the reference material were compared to vehicle group; S.E.M., standard error of the mean.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

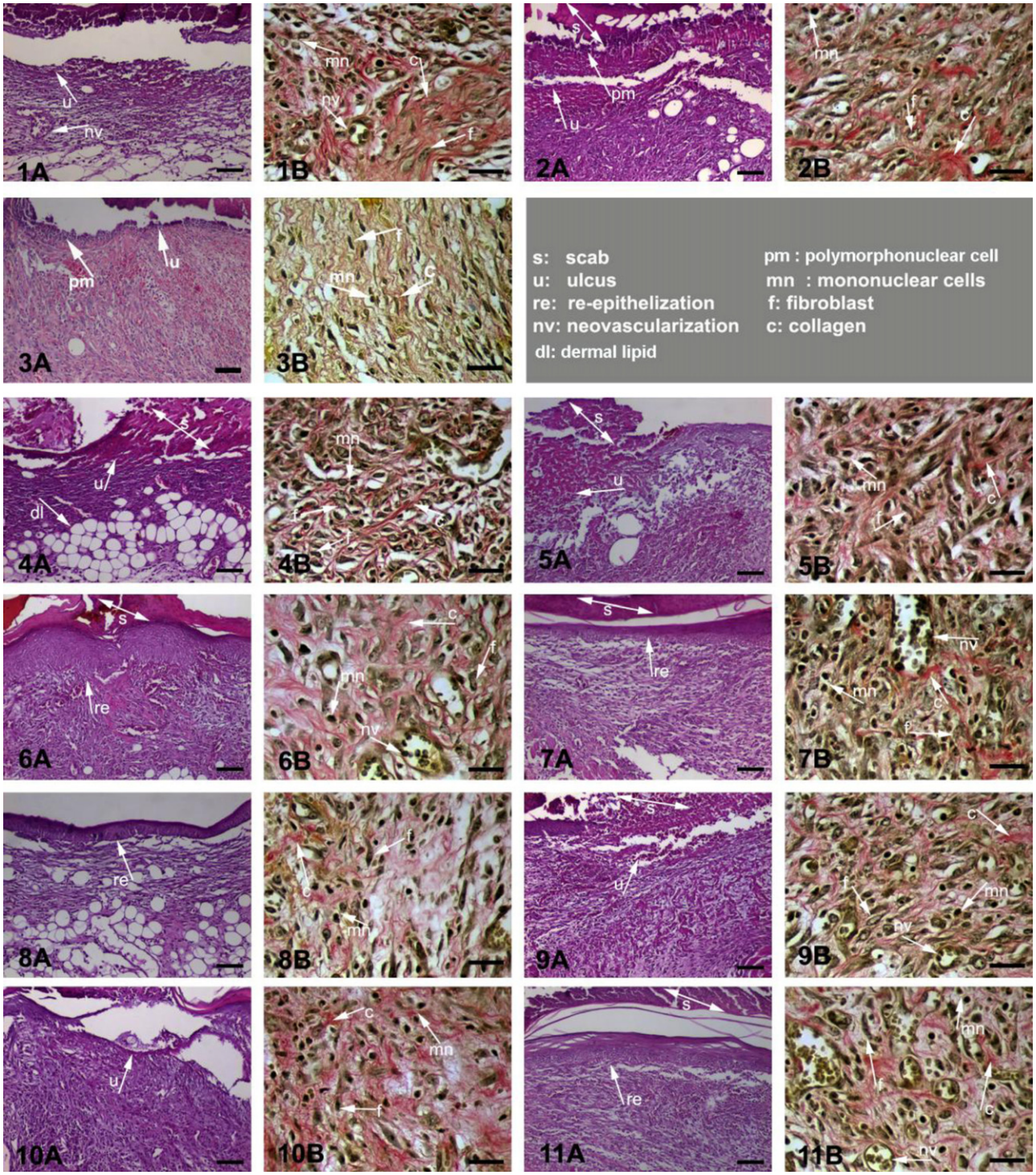

Fig. 4. Histopathological view of wound healing and epidermal/dermal re-modeling in the vehicle, negative control, extract, subextracts and reference ointment Madecassol® administered animals. Skin sections show the hematoxylin & eosin (HE) stained epidermis and dermis in (A), and the dermis stained with Van Gieson (VG) in (B). The original magnification was 100× and the scale bars represent 120 μm for figure in (A), and the original magnification was 400× and the scale bars represent 40 μm for (B). Data are representative of 6 animal per group. 1. Vehicle; 2. Negative control; 3. DOK-MeOH; 4. DOK-*n*-Hekzan; 5. DOK-CH_2Cl_2; 6. DOK-EtOAc (1%), 7. DOK-EtOAc (3%); 8. DOK-EtOAc (5%), 9. DOK-*n*-BuOH; 10. DOK-R-H_2O; 11. Madecassol. Arrows pointing events during wound healing; s: scab, u: ulcus, re: re-epithelialization, f: fibroblast, c: collagen, mnc: mononuclear cells, pmn: polymorphonuclear cells, nv: neovascularization.

is useful to determine the efficiency of test material against increased capillary permeability induced by intraperitoneal injection of acetic acid (Yesilada and Küpeli, 2007). Identical activity pattern was also observed in anti-inflammatory activity testing. DOK-EtOAc subextract, DOK-Fr. (22–39) and luteolin-7-*O*-glucoside exerted highest anti-inflammatory activity, provided 30.1%, 32.3% and 34.4% inhibition, respectively (Tables 10–12).

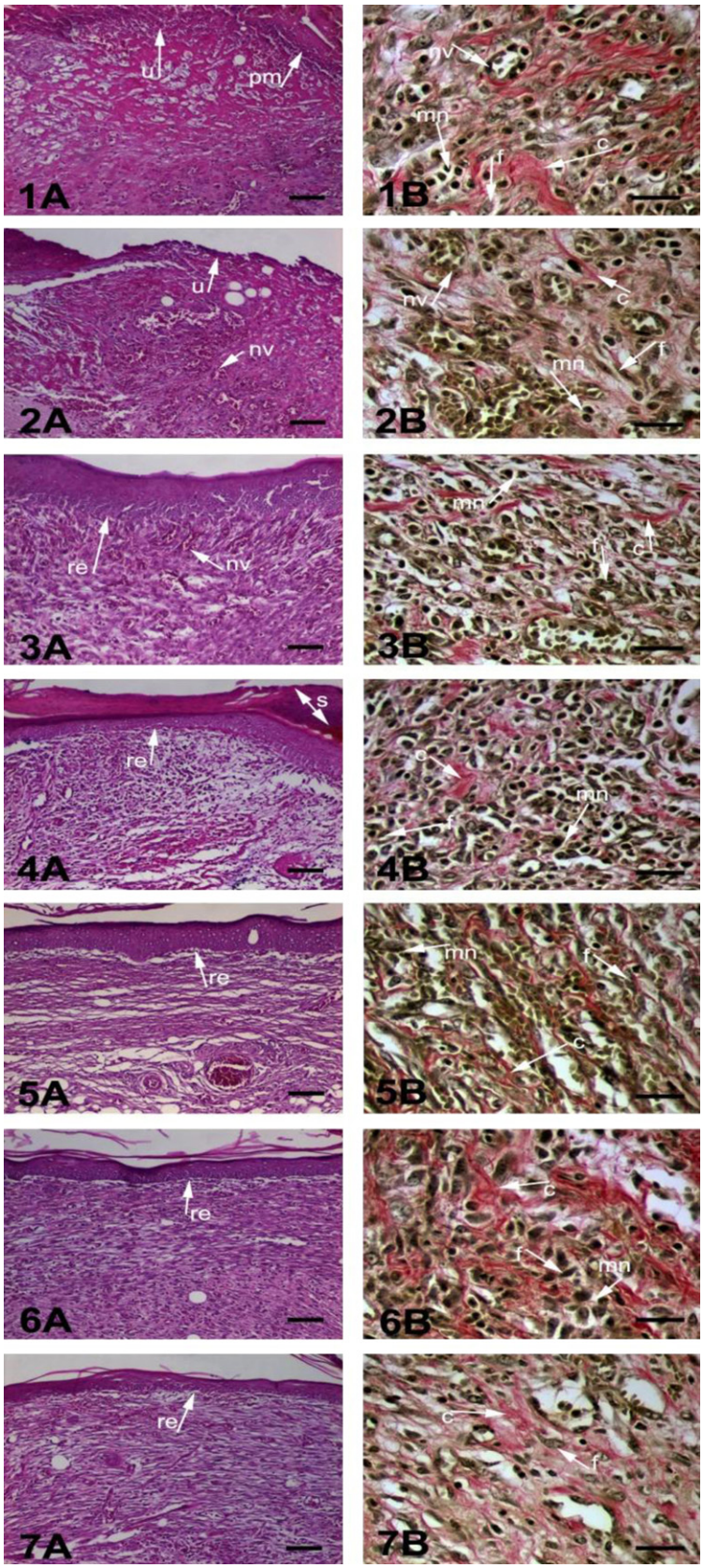

Fig. 5. Histopathological view of wound healing and epidermal/dermal re-modeling in the vehicle, negative control, fractions and reference ointment Madecassol® administered animals. Skin sections show the hematoxylin & eosin (HE) stained epidermis and dermis in (A), and the dermis stained with Van Gieson (VG) in (B). The original magnification was 100× and the scale bars represent 120 μm for figures in (A), and the original magnification was 400× and the scale bars represent 40 μm for (B). Data are representative of 6 animal per group. 1. Vehicle; 2. Negative control; 3. DOK-Fr. (1–8); 4. DOK-Fr. (9–15); 5. DOK-Fr. (16–21); 6. DOK-Fr. (22–39), 7. Madecassol. Arrows pointing events during wound healing; s: scab, u: ulcus, re: re-epithelialization, f: fibroblast, c: collagen, mnc: mononuclear cells, pmn: polymorphonuclear cells, nv: neovascularization.

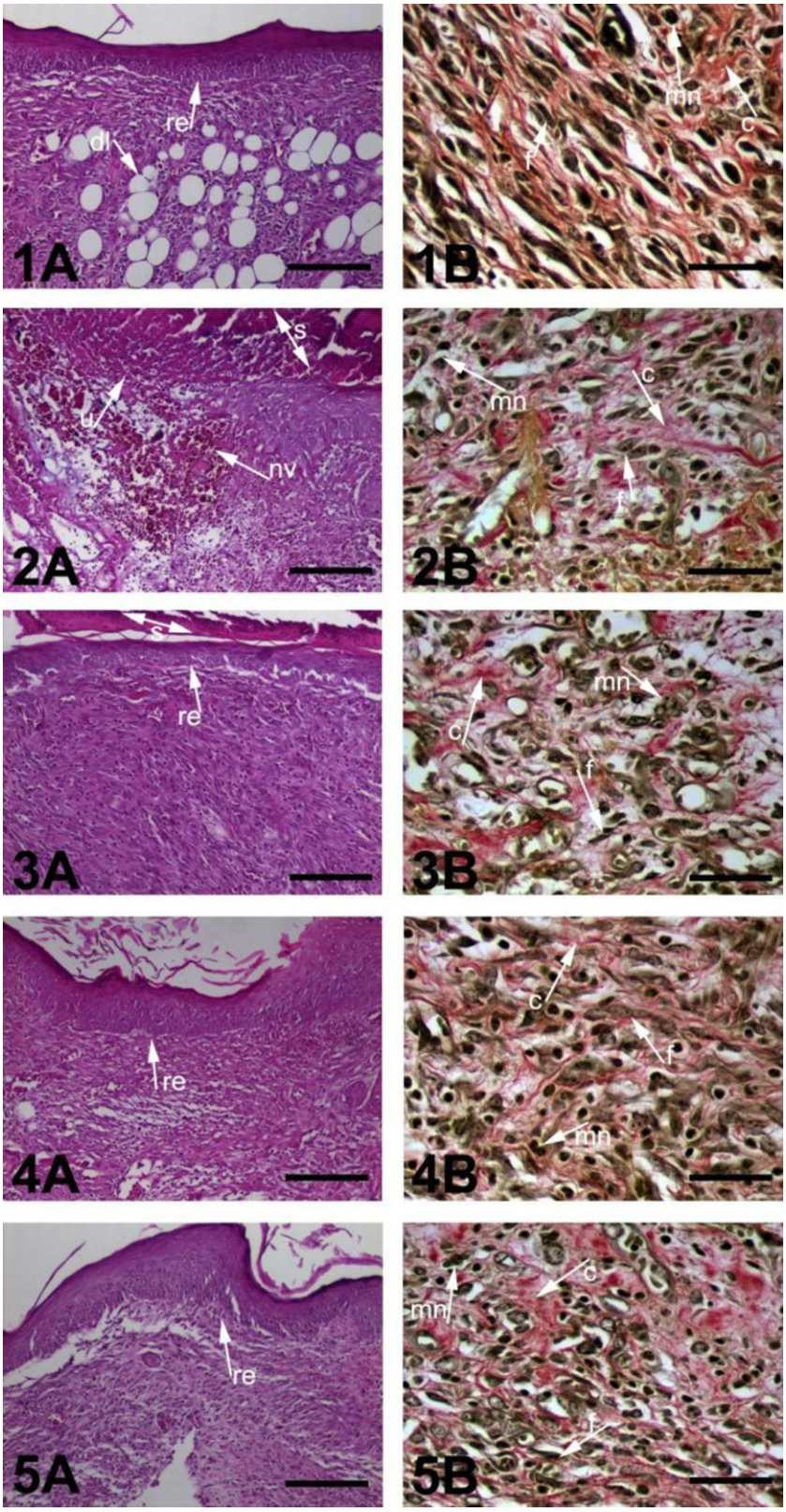

Fig. 6. Histopathological view of wound healing and epidermal/dermal re-modeling in the vehicle, negative control, isolated compounds and reference ointment Madecassol® administered animals. Skin sections show the hematoxylin & eosin (HE) stained epidermis and dermis in (A), and the dermis stained with Van Gieson (VG) in (B). The original magnification was 100× and the scale bars represent 120 μm for figure in (A), and the original magnification was 400× and the scale bars represent 40 μm for (B). Data are representative of 6 animal per group. 1. Vehicle; 2. Negative control; 3. Daphnetin; 4. Luteolin-7-*O*-glucoside; 5. Madecassol. Arrows pointing events during wound healing; s: scab, u: ulcus, re: re-epithelialization, f: fibroblast, c: collagen, mnc: mononuclear cells, pmn: polymorphonuclear cells, nv: neovascularization.

Table 5
Effects of the compounds from DOK-Fr. (22–39) on linear incision wound model.

Material	Tensile strength ± S.E.M.	(%Tensile strength)
Vehicle	8.85 ± 2.06	3.99
Negative control	8.51 ± 2.11	–
Daphnetin	10.06 ± 1.87	13.67
Luteolin-7-*O*-glucoside	12.38 ± 1.48	39.89**
Madecassol®	14.07 ± 0.95	58.98***

Percentage of the tensile strength values: the vehicle group was compared to the negative control group; the extracts and the reference material were compared to vehicle group; S.E.M., standard error of the mean.
** $p < 0.01$.
*** $p < 0.001$.

The antioxidant activity of the test materials was determined by the DPPH scavenging assay. The IC_{50} values of DOK-MeOH, EtOAc subextract, DOK-Fr. (22–39) and luteolin-3-*O*-glucoside were found to be highly comparable to those of the other extracts, except methylene chloride extract (Table 13). In spite of high total phenol content (Table 14) and the highest DPPH scavenging activity, methylene chloride extract did not show any remarkable wound-healing effect.

Furthermore, *in vitro* enzyme inhibitory activity tests revealed that active wound healing component luteolin-7-*O*-glucoside showed hyaluronidase and collagenase enzymes inhibitory activities with the values of 41.41% and 68.12%, respectively, while the compound did not show any elastase enzyme inhibition (Tables 15 and 16).

Previous studies showed that *Daphne oleoides* subsp. *oleoides*, *Daphne genkwa*, *Daphne pontica*, *Daphne retusa*, *Daphne gnidium* and *Daphne odora* have anti-inflammatory activities (Craker and Simon, 1987; Sakuma et al., 1998; Yesilada et al., 2001; Kupeli et al., 2007; Lee et al., 2009). Daphnetin (**1**) was previously isolated from the roots of *Daphne oleoides* subsp. *oleoides* as the anti-inflammatory principle through bioassay-guided technique and this compound was reported to exert inhibitory effect on the biosynthesis of IL-1α, IL-1β and TNF-α (Yesilada et al., 2001). On the other hand, luteolin and its glucosides were reported to possess antioxidant (Lemanska et al., 2004; Odontuya et al., 2005), antibacterial (Sato et al., 2000; Chung et al., 2001), antiviral (Yi et al., 2004; Wu et al., 2005), antifungal (Kırmızıbekmez et al., 2011), antiplasmodial (Harborne and Williams, 2000) and anticancerogenic (Peters et al., 1986; Plaumann et al., 1996) activities. The anti-inflammatory activity of luteolin glycosides were mainly exerted their activity through inhibition of nuclear factor-kappa B (NF-kappa B), cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX) and inducible nitric oxide synthase (iNOS) (Sadik et al., 2003; Harris et al., 2006).

Extracellular matrix (ECM) is composed of proteoglycans and matrix metalloproteins such as collagen and elastin. Collagen is the major structural protein provides supportive framework to the cell, elastin maintains the skin's elasticity and hyaluronic acid keeps the moist. All three components help the wound healing process (Sahasrabudhe and Deodhar, 2010). Therefore, inhibition of the enzymes that break down these ECM components could contribute wound healing. Therefore, luteolin-7-*O*-glucoside could probably show its wound healing effect by hyaluronidase and collagenase enzymes inhibition. The experimental results from the present investigation revealed that daphnetin did not show any significant wound healing effect. The possible anticoagulant effect of the coumarin type compounds could decrease the activity due to their hemostasis inhibitory effect. Hemostasis is an important step in the early response. As a result, it was concluded that flavonoid type compound, luteolin-7-*O*-glucoside isolated from DOK is responsible from the wound healing activity.

Table 6
Effects of the compounds from DOK-Fr. (22–39) on circular excision wound model.

Material	Wound area (mm^2) ± S.E.M. (Contraction%)						
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Vehicle	19.64 ± 2.10	16.76 ± 1.82 (7.66)	15.61 ± 1.24 (1.76)	13.29 ± 1.44 (7.41)	8.21 ± 1.16 (1.08)	4.83 ± 0.87 (5.66)	3.09 ± 0.62 (5.21)
Negative control	19.35 ± 2.31	18.15 ± 1.73	15.89 ± 1.32	13.49 ± 1.25	8.30 ± 1.28	5.12 ± 1.07	3.26 ± 0.68
Daphnetin	19.18 ± 2.13	16.22 ± 1.25 (3.22)	15.05 ± 1.14 (6.41)	13.12 ± 1.21 (1.28)	7.43 ± 1.36 (9.50)	3.95 ± 0.79 (18.22)	2.25 ± 0.36 (27.18)
Luteolin-7-*O*-glucoside	19.50 ± 2.04	15.92 ± 1.15 (5.01)	14.01 ± 1.16 (6.41)	11.12 ± 1.23 (16.33)	5.58 ± 1.05 (32.03)*	2.79 ± 0.16 (42.24)*	1.20 ± 0.12 (61.17)**
Madecassol®	19.24 ± 2.17	15.16 ± 1.20 (9.55)	12.57 ± 1.10 (19.47)	8.03 ± 0.51 (39.58)*	3.41 ± 0.56 (58.47)**	1.18 ± 0.29 (75.57)**	0.00 ± 0.00 (100.00)***

Percentage of the contraction values: the vehicle group was compared to the negative control group; the fractions and the reference material were compared to vehicle group; S.E.M., standard error of the mean.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 7
Effects of the test ointments prepared from the extract and sub-extracts of *Daphne oleoides* subsp. *kurdica* on hydroxyproline content.

Material	Hydroxyproline (μg/mg) ± S.E.M.
Vehicle	9.15 ± 1.64
Negative control	7.72 ± 1.10
DOK-MeOH	31.31 ± 1.36***
DOK-*n*-hexane	19.83 ± 1.74
DOK-CH_2Cl_2	22.70 ± 2.20
DOK-EtOAc	32.77 ± 0.86***
DOK-*n*-BuOH	21.12 ± 1.96
DOK-R-H_2O	10.37 ± 1.29
Madecassol®	40.14 ± 0.63***

S.E.M., standard error of the mean.
*** $p < 0.001$.

Table 8
Effects of the test ointments prepared from the fractions of DOK-EtOAc on hydroxyproline content.

Material	Hydroxyproline (μg/mg) ± S.E.M.
Vehicle	8.27 ± 1.73
Negative control	5.49 ± 1.80
DOK-Fr. (1–8)	11.13 ± 2.62
DOK-Fr. (9–15)	16.36 ± 1.22
DOK-Fr. (16–21)	13.15 ± 1.77
DOK-Fr. (22–39)	25.12 ± 0.89**
Madecassol®	44.18 ± 0.76***

S.E.M., standard error of the mean.
** $p < 0.01$.
*** $p < 0.001$.

Table 9
Effects of the test ointments prepared from the compounds of DOK-Fr. (22–39) on hydroxyproline content.

Material	Hydroxyproline (μg/mg) ± S.E.M.
Vehicle	12.35 ± 1.45
Negative control	10.91 ± 1.36
Daphnetin	21.76 ± 1.81
Luteolin-7-*O*-glucoside	40.33 ± 1.01***
Madecassol®	49.39 ± 0.58***

S.E.M., standard error of the mean.
*** $p < 0.001$.

Table 10
Effects of the extract and sub-extracts from *Daphne oleoides* subsp. *kurdica* on Whittle method.

Material	Dose (mg/kg)	Evans blue concentration (μg/mL) ± S.E.M.	Inhibition (%)
Control		11.28 ± 0.59	
DOK-MeOH	100	14.07 ± 0.32	24.73*
	200	15.71 ± 0.66	39.27**
DOK-*n*-hexane	100	11.35 ± 0.85	–
	200	10.75 ± 0.76	4.69
DOK-CH_2Cl_2	100	11.12 ± 0.73	1.42
	200	10.91 ± 0.74	3.28
DOK-EtOAc	100	9.20 ± 0.91	18.44
	200	7.88 ± 0.31	30.14**
DOK-*n*-BuOH	100	9.55 ± 0.68	15.34
	200	8.65 ± 0.46	23.32*
DOK-R-H_2O	100	10.85 ± 0.64	3.81
	200	10.13 ± 0.59	10.19
Indomethacin	10.0	6.65 ± 0.39	41.05***

S.E.M., standard error of the mean.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 11
Effects of the fractions from DOK-EtOAc on Whittle method.

Material	Dose (mg/kg)	Evans blue concentration (μg/mL) ± S.E.M.	Inhibition (%)
Control		9.96 ± 0.81	
DOK-Fr. (1–8)	200	8.59 ± 0.79	13.76
DOK-Fr. (9–15)	200	11.07 ± 0.96	–
DOK-Fr. (16–21)	200	9.10 ± 0.88	8.63
DOK-Fr. (22–39)	200	6.74 ± 0.62	32.33**
Indomethacin	10.0	4.44 ± 0.43	55.42***

S.E.M., standard error of the mean.
** $p < 0.01$.
*** $p < 0.001$.

Table 12
Effects of the compounds from DOK-Fr. (22–39) on Whittle method.

Material	Dose (mg/kg)	Evans blue concentration (μg/mL) ± S.E.M.	Inhibition (%)
Control		10.43 ± 0.72	
Daphnetin	200	8.11 ± 0.84	22.24
Luteolin-7-*O*-glucoside	200	6.84 ± 0.44	34.42**
Indomethacin	10.0	5.06 ± 0.36	51.50***

S.E.M., standard error of the mean.
** $p < 0.01$.
*** $p < 0.001$.

Table 13
The DPPH scavenging activity of the extract, sub-extracts, fractions and compounds from *Daphne oleoides* subsp. *kurdica*.

Material	IC_{50} (μg/mL)
DOK-MeOH	95.98
DOK-*n*-hexane	103.81
DOK-CH_2Cl_2	10.61
DOK-EtOAc	26.70
DOK-BuOH	627.36
DOK-R-H_2O	1560.84
DOK-Fr. (1–8)	43.51
DOK-Fr. (9–15)	37.28
DOK-Fr. (16–21)	67.73
DOK-Fr. (22–39)	29.35
Daphnetin	27.12
Luteolin-7-*O*-glucoside	18.21
Reference (Quercetin)	2.14

Table 14
The total phenolic content of the extract and sub-extracts from *Daphne oleoides* subsp. *kurdica*.

Extracts and sub-extracts	Total phenolics (mg GA/g ± S.E.M.)
DOK-MeOH	117.43 ± 1.25
DOK-*n*-hexane	7.42 ± 1.13
DOK-CH_2Cl_2	191.00 ± 1.36
DOK-EtOAc	221.00 ± 1.01
DOK-*n*-BuOH	101.71 ± 1.28
DOK-R-H_2O	42.6 ± 1.53

S.E.M., standard error of the mean.

Table 15
Hyaluronidase enzyme inhibitory activity of the isolated compounds from *Daphne oleoides* subsp. *kurdica*.

Material	Concentration (μg/mL)	Inhibition (%) ± S.E.M.
Daphnetin	50	8.29 ± 1.13
	100	18.34 ± 1.08
Luteolin-7-*O*-glucoside	50	29.10 ± 1.27
	100	41.41 ± 0.96*
Tannic acid	100	85.24 ± 0.82***

S.E.M., standard error of the mean.
* $p < 0.05$.
*** $p < 0.001$.

Table 16
Collagenase and elastase enzyme inhibitory activities of the isolated compounds from *Daphne oleoides* subsp. *kurdica*.

Material	Concentration (μg/mL)	Collagnease inhibition (%) ± S.E.M.	Elastase inhibition (%) ± S.E.M.
Daphnetin	50	14.25 ± 1.18	15.23 ± 1.16
	100	27.15 ± 1.21	18.10 ± 1.26
Luteolin-7-*O*-glucoside	50	28.63 ± 1.04	20.24 ± 1.43
	100	68.12 ± 0.71**	32.11 ± 1.35
Epigallocatechin gallate	100	39.08 ± 0.98**	85.05 ± 1.04***

S.E.M., standard error of the mean.
** $p < 0.01$.
*** $p < 0.001$.

4. Conclusion

In conclusion, by using bioassay-guided processing luteolin-7-*O*-glucoside was isolated as the active wound-healing component of DOK. The wound-healing activity mechanism of this flavonoid type compound may be attributed to its significant anti-inflammatory, antioxidant, and hyaluronidase and collagenase enzyme inhibitory activities together.

Acknowledgements

This study was supported by 2214-International Doctoral Research Fellowship Programme, provided by "The Scientific and Technological Research Council of Turkey (TUBITAK)" and the Scientific Research Project of Gazi University, Grant No.: 02/2010-15.

We thank EPSRC National Mass Spectrometry Service Centre, Department of Chemistry, University of Wales Swansea, Swansea, Wales, UK.

References

Baba, K., Takeuchi, K., Hamasaki, F., Kozawa, M., 1986. Chemical studies on the constituents of the Thymelaeaceous plants. I. Structure of two new flavans from *Daphne odora* Thunb. Chemical and Pharmaceutical Bulletin 34, 595–602.

Barrantes, E., Guinea, M., 2003. Inhibition of collagenase and metalloproteinases by aloins and aloe gel. Life Sciences 72, 843–850.

Baytop, T., 1999. Therapy with Medicinal Plants in Turkey. İstanbul University Press, İstanbul.

Chung, J.G., Hsia, T.C., Kuo, H.M., Li, Y.C., Lee, Y.M., Lin, S.S., Hung, C.F., 2001. Inhibitory actions of luteolin on the growth and arylamine N-acetyltransferase activity in strains of *Helicobacter pylori* from ulcer patients. Toxicology In Vitro 15, 191–198.

Cottiglia, F., Loy, G., Garau, D., Floris, C., Casu, M., Pompei, R., Bonsignore, L., 2001. Antimicrobial evaluation of coumarins and flavonoids from the stems of *Daphne gnidium* L. Phytomedicine 8, 302–305.

Craker, L.E., Simon, J.E., 1987. Herbs, Species and Medicinal Plants: Recent Advances in Botany, Horticulture and Pharmacology, vol. 2. Oryx Press, Canada.

Degim, Z., Çelebi, N., Sayan, H., Babül, A., Erdoğan, D., Take, G., 2002. An investigation on skin wound healing in mice with a taurine-chitosan gel formulation. Amino Acids 22, 187–198.

Deiana, M., Rosa, A., Casu, V., Cottiglia, F., Bonsignore, L., Dessi, M.A., 2003. Chemical composition and antioxidant activity of extracts from *Daphne gnidium* L. Journal of American Oil Chemists Society 80, 65–70.

Fujita, T., Sezik, E., Tabata, M., Yesilada, E., Honda, G., Takeda, Y., Tanaka, T., Takaishi, Y., 1995. Traditional medicine in Turkey VII. Folk Medicine in Middle and West Black Sea Regions. Economic Botany 49, 406–422.

Gohari, A.R., Ebrahimi, H., Saeidnia, S., Foruzani, M., Ebrahimi, P., Ajani, Y., 2011. Flavones and flavone glycosides from *Salvia macrosiphon* Boiss. Iranian Journal of Pharmaceutical Research 10, 247–251.

Grieve, M., Leyel, C.F., 1967. A Modern Herbal, The Medicinal, Culinary, Cosmetic and Economic Properties, Cultivation and Folklore of Herbs, Grasses, Fungi; Shrubs and Trees, vol. 2. Herner Publishing Co., New York.

Harborne, J.B., Williams, C.A., 2000. Advances in flavonoid research since 1992. Phytochemistry 55, 481–504.

Harris, G.K., Qian, Y., Leonard, S.S., Sbarra, D.C., Shi, X., 2006. Luteolin and chrysin differentially inhibit cyclooxygenase-2 expression and scavenge reactive oxygen species but similarly inhibit prostaglandin-E2 formation in RAW 264.7 cells. Journal of Nutrition 136, 1517–1521.

Hong, C.H., Hur, S.K., Oh, O.J., Kim, S.S., Nam, K.A., Lee, S.K., 2002. Evaluation of natural products on inhibition of inducible cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) in cultured mouse macrophage cells. Journal of Ethnopharmacology 82, 153–159.

Kırmızıbekmez, H., Atay, I., Kaiser, M., Brun, R., Cartagena, M.M., Carballeira, N.M., Yesilada, E., Tasdemir, D., 2011. Antiprotozoal activity of *Melampyrum arvense* and its metabolites. Phytotherapy Research 25 (1), 142–146.

Kreher, B., Neszmelyi, A., Wagner, H., 1990. Triumbellin, a tricoumarin rhamnopyranoside from *Daphne mezereum*. Phytochemistry 29 (11), 3633–3637.

Krishnan, P., 2006. The scientific study of herbal wound healing therapies: current state of play. Current Anaesthesia and Critical Care 17, 21–27.

Kumar, B., Vijayakumar, M., Govindarajan, R., Pushpangadan, P., 2007. Ethnopharmacological approaches to wound healing-exploring medicinal plants of India. Journal of Ethnopharmacology 114, 103–113.

Kumarasamy, Y., Nahar, L., Cox, P.J., Jaspars, M., Sarker, S.D., 2003. Bioactivity of secoiridoid glycosides from *Centaurium erythraea*. Phytomedicine 10, 344–347.

Kupeli, E., Tosun, A., Yesilada, E., 2007. Assessment of anti-inflammatory and antinociceptive activities of *Daphne pontica* L. (Thymelaeaceae). Journal of Ethnopharmacology 113, 332–337.

Lee, K.K., Choi, J.D., 1999. The effects of *Areca catechu* L. extracts on anti ageing. International Journal of Cosmetic Sciences 21, 285–294.

Lee, M.Y., Park, B.Y., Kwon, O.K., Yuk, J.E., Oh, S.R., Kim, H.S., Lee, H.K., Ahn, K.S., 2009. Anti-inflammatory activity of (−)-aptosimon isolated from *Daphne genkwa* in RAW264.7 cells. International Immunopharmacology 9, 878–885.

Lemanska, K., Van der, W.H., Szymusiak, H., Boersma, M.G., Gliszczynska-Swiglo, A., Rietjens, I.M., Tyrakowska, B., 2004. The effect of catechol O-methylation on radical scavenging characteristics of quercetin and luteolin—a mechanistic insight. Free Radical Research 38, 639–647.

Lodhi, S., Pawar, R.S., Jain, A.P., Singhai, A.K., 2006. Wound healing potential of *Tephrosia purpurea* (Linn.) Pers. in rats. Journal of Ethnopharmacology 108, 204–210.

Ma, B., Guo, H.F., Lou, H.X., 2007. A new lignan and two eudesmanes from *Lepidozia vitrea*. Helvetica Chimica Acta 90, 58–62.

Melzig, M.F., Löser, B., Ciesielski, S., 2001. Inhibition of neutrophil elastase activity by phenolic compounds from plants. Pharmazie 56, 967–970.

Murakami, S., Arai, I., Muramatsu, M., Otomoto, S., Baba, K., Kozawa, M., 1992. Daphnodorins inhibit gastric H^+,K^+-ATPase and acid secretion. Pharmaceutical and Pharmacological Letters 2, 108–111.

Murray, R.D., Mendez, J., Brown, S.A., 1982. The Natural Coumarins, Occurrence, Chemistry and Biochemistry. John Wiley and Sons Ltd., New York.

Niwa, M., Sugino, H., Takashima, S., Sakai, T., Wu, Y.C., Wu, T.S., Kuoh, C.S., 1991. A new coumarin glucoside from *Daphne arisanensis*. Chemical and Pharmaceutical Bulletin 39, 2422–2424.

Odontuya, G., Hoult, J.R., Houghton, P.J., 2005. Structure–activity relationship for anti-inflammatory effect of luteolin and its derived glycosides. Phytotherapy Research 19, 782–786.

Okunishi, T., Takaku, N., Wattanawikkit, P., Sakakibara, N., Suzuki, S., Sakai, F., Umezawa, T., Shimada, M., 2002. Lignan production in *Daphne odora* cell cultures. Journal of Wood Science 48, 237–241.

Peters, N.K., Frost, J.W., Long, S.R., 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science 233, 977–980.

Plaumann, B., Fritsche, M., Rimpler, H., Brandner, G., Hess, R.D., 1996. Flavonoids activate wild-type p53. Oncogene 13, 1605–1614.

Riaz, M., Krohn, K., Wray, V., Malik, A., 2002. Dicoumarinyl ether glycoside from the roots of *Daphne oleoides*. European Journal of Organic Chemistry 8, 1436–1438.

Ronlan, A., Wickberg, B., 1970. The structure of *mezerein*, a major toxic principle of *Daphne mezereum* L. Tetrahedron Letters 49, 4261–4264.

Sadik, C.D., Sies, H., Schewe, T., 2003. Inhibition of 15-lipoxygenases by flavonoids: structure–activity relations and mode of action. Biochemical Pharmacology 65, 773–781.

Sahasrabudhe, A., Deodhar, M., 2010. Anti-hyaluronidase, anti-elastase activity *of Garcinia indica*. International Journal of Botany 6, 299–303.

Sakuma, S., Fujimoto, Y., Tsunomori, M., Tagano, S., Nishida, H., Baba, K., Fujita, T., 1998. Effects of daphnodorin A, B and C, new flavans isolated from traditional Chinese medicine, on the 12-lipoxigenase and cyclooxygenase metabolism of arachidonic acid in rabbit platelets. Prostaglandins Leukotrienes and Essential Fatty Acids 58, 143–146.

Sato, Y., Suzaki, S., Nishikawa, T., Kihara, M., Shibata, H., Higuti, T., 2000. Phytochemical flavones isolated from *Scutellaria barbata* and antibacterial activity against methicillin-resistant *Staphylococcus aureus*. Journal of Ethnopharmacology 72, 483–488.

Spanos, G.A., Wrolstad, R.E., 1990. Influence of processing and storage on the phenolic composition of Thompson seedless grape juice. Journal of Agricultural Food and Chemistry 38, 1565–1571.

Suguna, L., Singh, S., Sivakumar, P., Sampath, P., Chandrakasan, G., 2002. Influence of *Terminalia chebula* on dermal wound healing in rats. Phytotherapy Research 16, 227–231.

Süntar, I., Baldemir, A., Coşkun, M., Keleş, H., Küpeli Akkol, E., 2011. Wound healing acceleration effect of endemic *Ononis* species growing in Turkey. Journal of Ethnopharmacology 135, 63–70.

Tan, K., 1982. Daphne. In: Davis, P.H. (Ed.), Flora of Turkey and the East Aegean Islands, vol. 7. The University Press, Edinburgh.

Taninaka, H., Takaishi, Y., Honda, G., Imakura, Y., Sezik, E., Yesilada, E., 1999. Terpenoids and aromatic compounds from *Daphne oleoides* subsp. *oleoides*. Phytochemistry 52, 1525–1529.

Tramontina, V.A., Machado, M.A., Nogueira Filho Gda, R., Kim, S.H., Vizzioli, M.R., Toledo, S., 2002. Effect of bismuth subgallate (local hemostatic agent) on wound

healing in rats. Histological and histometric findings. Brazilian Dental Journal 13, 11–16.
Ulubelen, A., Terem, B., Tuzlacı, E., 1986. Coumarins and flavonoids from *Daphne gnidioides*. Journal of Natural Products 49, 692–694.
Ullah, N., Ahmed, Z., Anis, E., Malik, A., 1998. Phytochemical investigation of *Daphne oleoides*. Fitoterapia 69, 280.
Ullah, N., 1999. Phytochemical investigation of *Daphne oleoides*. Doctor of Philosophy Thesis, University of Karachi, Karachi.
Whittle, B.A., 1964. The use of changes in capillary permeability in mice to distinguish between narcotic and non-narcotic analgesics. British Journal of Pharmacology and Chemotherapy 22, 246–253.
Wu, M.J., Weng, C.Y., Ding, H.Y., Wu, P.J., 2005. Anti-inflammatory and antiviral effects of *Glossogyne tenuifolia*. Life Sciences 76, 1135–1146.
Yesilada, E., Honda, G., Sezik, E., Tabata, M., Fujita, T., Tanaka, T., Takeda, Y., Takaishi, Y., 1995. Traditional medicine in Turkey. V. Folk medicine in the inner Taurus Mountains. Journal of Ethnopharmacology 46, 133–152.
Yesilada, E., Sezik, E., Honda, G., Takaishi, Y., Takeda, Y., Tanaka, T., 1999. Traditional medicine in Turkey IX. Folk medicine in North-west Anatolia. Journal of Ethnopharmacology 64, 195–210.
Yesilada, E., Taninaka, H., Takaishi, Y., Honda, G., Sezik, E., Momota, H., Ohmoto, Y., Taki, T., 2001. *In vitro* inhibitory effects of *Daphne oleoides* subsp. *oleoides* on inflammatory cytokines and activity-guided isolation of active constituents. Cytokine 13, 359–364.
Yesilada, E., Küpeli, E., 2007. *Clematis vitalba* L. aerial part exhibits potent anti-inflammatory, antinociceptive and antipyretic effects. Journal of Ethnopharmacology 110, 504–515.
Yi, L., Li, Z., Yuan, K., Qu, X., Chen, J., Wang, G., Zhang, H., Luo, H., Zhu, L., Jiang, P., Chen, L., Shen, Y., Luo, M., Zuo, G., Hu, J., Duan, D., Nie, Y., Shi, X., Wang, W., Han, Y., Li, T., Liu, Y., Ding, M., Deng, H., Xu, X., 2004. Small molecules blocking the entry of severe acute respiratory syndrome coronavirus into host cells. Journal of Virology 78, 11334–11339.
Zhang, Y., Zhang, H., Hua, S., Ma, L., Chen, C., Liu, X., Jiang, L., Yang, H., Zhang, P., Yu, D., Guo, Y., Tan, X., Liu, J., 2007. Identification of two herbal compounds with potential cholesterol-lowering activity. Biochemical Pharmacology 74, 940–947.
Zheng, W., Gao, X., Chen, C., Tan, R., 2007. Total flavonoids of *Daphne genkwa* root significantly inhibit the growth and metastasis of Lewis lung carcinoma in C57BL6 mice. International Immunopharmacology 7, 117–127.